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(71) Applicant : **PHARMA MAR, S.A.**
Calle de la Calera 3,
Poligono Industrial de Tres Cantos
E-28760 Tres Cantos, Madrid (ES)

(72) Inventor : **Schauer, Paul J.**
University of Hawaii,
Manoa, Honolulu,
Hawaii (US)
Inventor : **Hamann, Mark T.**
University of Hawaii,
Manoa, Honolulu
Hawaii (US)
Inventor : **Gravalos, Dolores G. c/o Pharma Mar, S.A.,**
Calle de La Calera No.3,
Poligono de Tres Cantos
28760 Tres Cantos, Madrid (ES)

(74) Representative : **Lamb, John Baxter**
MARKS & CLERK,
57-60 Lincoln's Inn Fields
London WC2A 3LS (GB)

(54) Cytotoxic and antiviral compound.

(57) Kalahide F, of formula I below, may be isolated from a sacoglossan. The compound may be used in the manufacture of pharmaceutical compositions or in the treatment of tumors or viral conditions.

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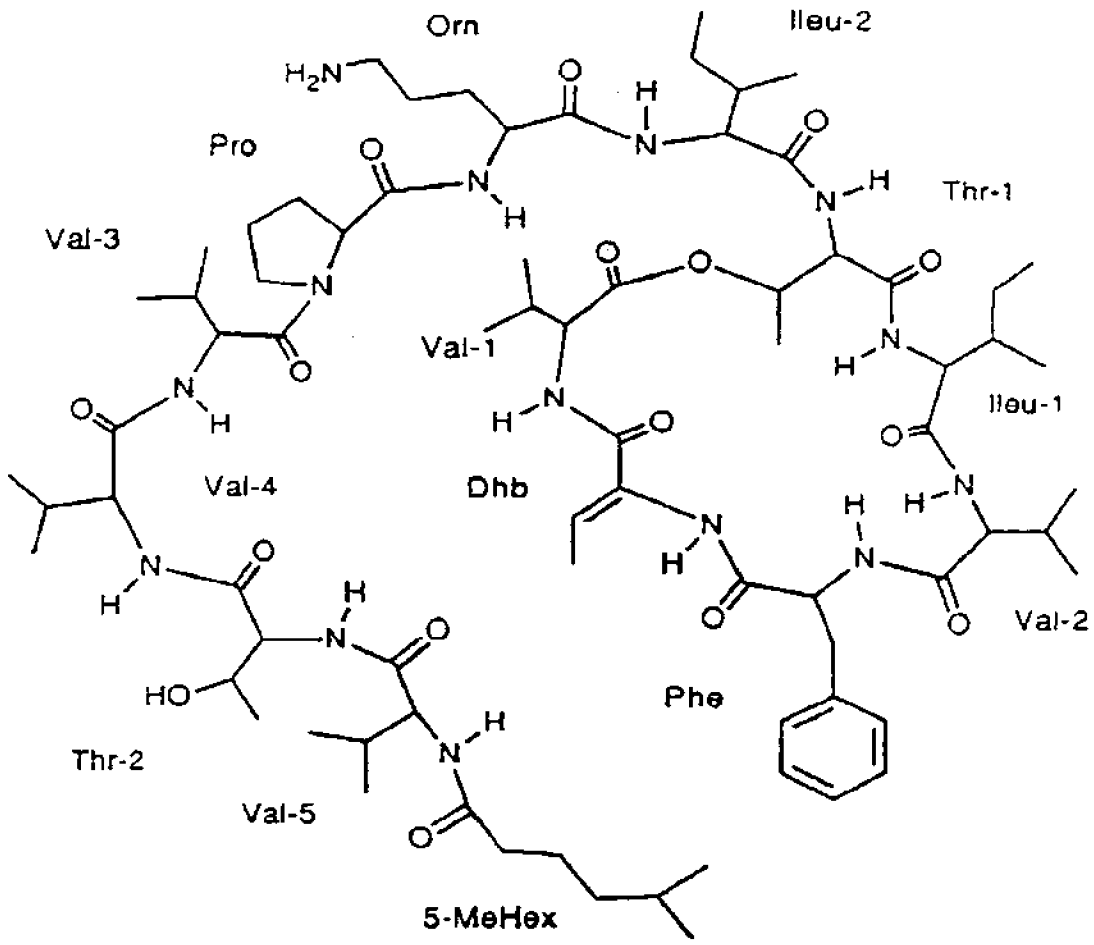
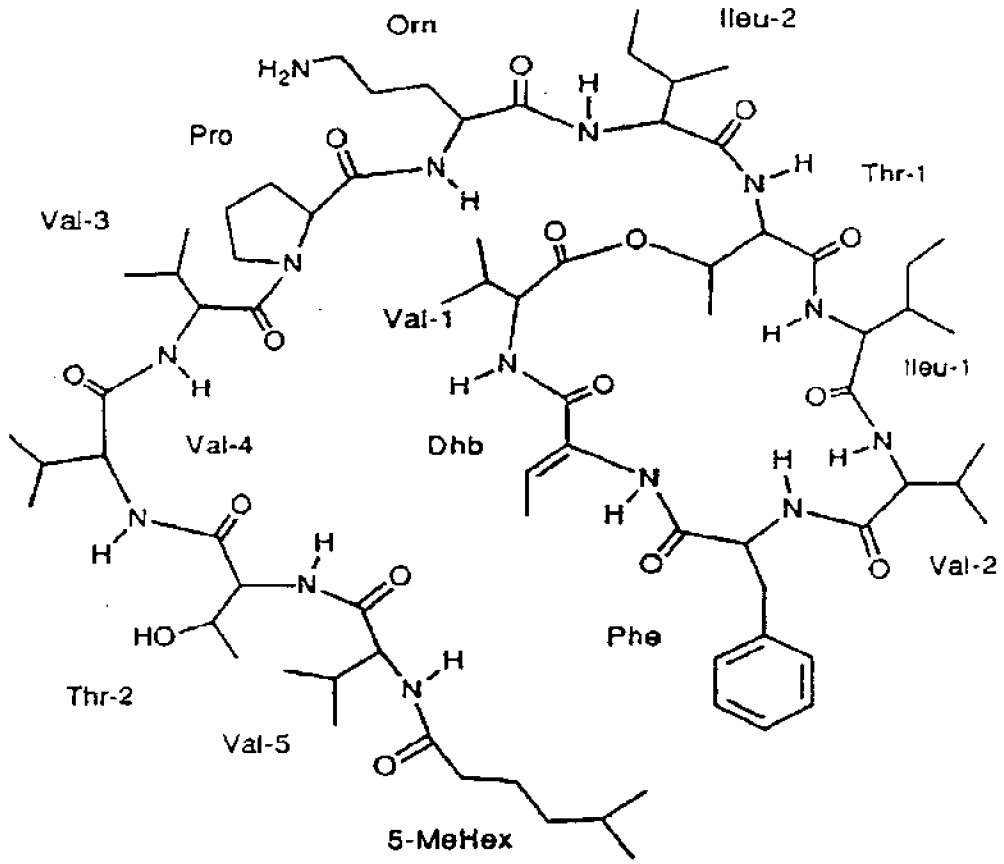


This invention is concerned with a cytotoxic and antiviral compound isolated from the sacoglossan, Elysia rafescens.

According to the invention there is provided, a new compound, the peptide, Kalahide F, of the formula:



The antitumor activities of this compound has been determinated "in vitro" in cell cultures of human lung carcinoma A-549 and human colon carcinoma HT-29. The procedure was carried out using the methodology described by Raymond J. Bergeron et al. *Biochem. Bioph. Res. Comm. 1984,* 121(3), 848-854 and by Alan C. Schroeder et al. *J. Med. Chem.* 1981, 24 1078-1083.

The antiviral activities of this compound have also been determinated "in vitro" against HSV (Herpes simplex virus) and VSV (Vesicular stomatitis virus). The methodology used to carry out this determination is described by Raymond J. Bergeron et al. *Biochem. Bioph. Res. Comm. 1984,* 121(3), 848-854 and by Alan C. Schroeder et al. *J. Med. Chem.* 1981, 24 1078-1083.

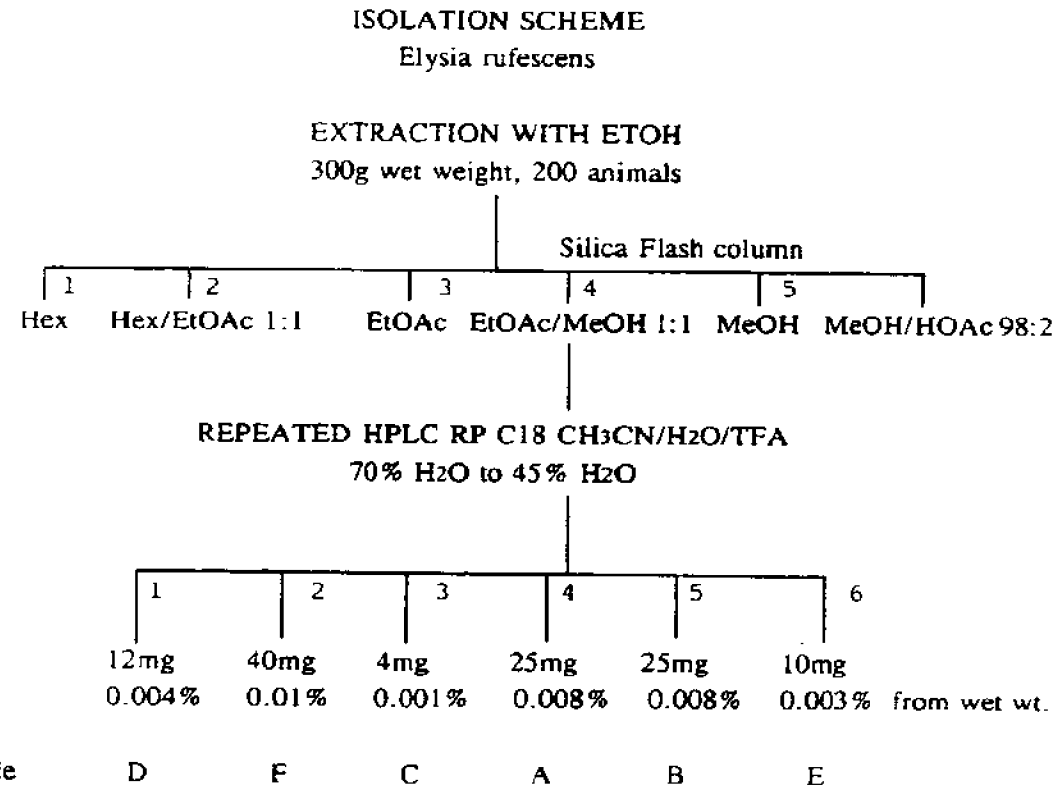
Therefore, the present invention also provides a method of treating any mammal affected by a malignant tumor sensitive to compounds above described, which comprises administering to the affected individual a therapeutically effective amount of these compounds or a pharmaceutically composition thereof; and a method of treating viral infections in mammals, comprising administering to a patient in need of such treatment, an antiviral effective amount of the compounds described in the present invention.

The present invention also relates to pharmaceutical preparations which contain as active ingredient these compounds, or a pharmaceutical acceptable acid addition salt thereof, as well as the process for its preparation.

Examples of pharmaceutical compositions include any solid(tablets, pills, capsules, granules, etc.) or liquid(solutions, suspensions or emulsions) suitable composition for oral, topical or parenteral administration, and they may contain the pure compound or in combination with any carrier or other pharmacologically active

compounds. These compositions may need to be sterile when administered parenterally.

The correct dosag of a pharmaceutical composition of these compounds will vary according to the particular formulation, the mode of application and particular situs, host and tumor being treat d. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of disease shall be taken in account. Administration can be carried out continuously or periodically within the maximum tolerated dose. Kahalalide F was isolated from the sacoglossan, Elysia rufescens (family Plakobranchidae, order Sacoglossa), collected near Black point, Oahu. This animal varies in size between 1 and 4 cm; it is dark red-brown in color with light-colored spots. There is orang fringing of the parapodia, which have very small dark green spots from sequestered chloroplasts. Elysia rufescens feeds on the delicate, feather-like green alga *Bryopsis sp.*[1] Kahalalide F can also be isolated from this alga. Two hundred animals were collected over the period of several weeks during spring, 1991 and extracted with EtOH. The extracts were then chromatographed by silica gel flash chromatography (hexane, hexane/EtOAc (1:1), EtOAc, EtOAc (1:1), MeOH and MeOH/HOAc (98:2). The peptides were eluted with EtOAc/MeOH (1:1). Final purification was accomplished by repeated HPLC (RP C18) using MeCN/H_2O with 0.1% TFA (70-45% H_2O) (Figure 1).



The structures of the peptides were elucidated by 2D NMR experiments (HMQC, HMBC, TOCSY, COSY and ROESY).

Kahalalide F was isolated as a white amorphous powder in 0.02% yield. A molecular formula of $C_{75}H_{124}N_{14}O_{16}$ was deduced from detailed analyses of the ^{13}C and ^{1}H NMR spectra and the high resolution FAB mass spectrum. The 14 substructures in this compound arise from five valines, two isoleucines, two threonines, ornithine, dehydroaminobutyric acid, proline, phenilalanine and 5-methylhexanoic acid (5-MeHex). Kahalalide F is the largest peptid in this seri s of compounds.

EXPERIMENTAL

General Considerations

Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Infrared spectra were recorded on a Nicolet MX-5 FTIR spectrometer. Gas chromatography was accomplished using a Hewlett-Packard Model 5890 instrument. Mass spectra were measured on a VG-70SE magnetic sector mass spectrometer. NMR spectra were measured on a General Electric QE-300 or a GN OMEGA 500 instrument. ^1H NMR chemical shifts are reported in ppm with the chemical shift of the residual protons of the solvent used as internal standards. ^{13}C NMR chemical shifts are reported in ppm by using the natural abundance ^{13}C of the solvent as an internal standard. Ultraviolet spectra were recorded on a Hewlett-Packard Model 8452A diode array spectrophotometer. All solvents were destilled from glass before use.

Two hundred sacoglossans (Elysia rufescens, Fig. 33) were collected at Black Point, O'ahu during April and May 1992, and extracted 3 times with EtOH. Spring appears to be the time of year Elysia rufescens is in greatest abundance at Black Point. The combined extracts were then chromatographed using silica gel flash chromatography (hexane, hexane/EtOAc (1:1), EtOAc, EtOAc/MeOH (1:1), MeOH, MeOH/HOAc (98:2). The depsipeptides were found in the EtOAc/MeOH (1:1) fraction. Repeated HPLC RP18 MeCN/H_2O/TFA (55/45/1) - MeCN/H_2O/TFA ((30/70/1) yielded six new depsipeptides. For details see Fig. 1.

KAHALALIDE F

Final purification was accomplished by HPLC on RP18 MeCN/H_2O/TFA (55/45/ 1).Physical data: [α]D-8°(*c* 4.32, MeOH); ^1H NMR (500 MHz, TFA/DMF); amino acid unit, δ (carbon position, mult, J): *Val-1* 4.16 (2, t, J=9.0 Hz), 7.11 (NH on 2, d, J=8.9 Hz), 1.77 (3, m), 0.95 (4, m), 0.95 (5, m); *Dhb* 9.20 (NH on 2, s), 6.48 (3, q, J=6.9 Hz), 1.43 (4, d, J=6.6 Hz); *Phe* 4.68 (2, q, J=6.6 Hz), 8.62 (NH on 2, d, J=6.6 Hz), 3.2 (3, dd, J=13.7, 7.2 Hz), 3.0(3, dd, J=13.7, 9.0 Hz), 7.32 (5, d, J=7.2 Hz), 7.28 (6, t, J=7.5 Hz), 7.21 (7, t, J=7.2 Hz); *Val-2* 4.36 (2, m), 7.82 (NH on 2, d, J=6.6 Hz), 2.12 (3, m), 0.85 (4, m), 0.77 (5, d, J=6.6 Hz); *Ileu-1* 4.53 (2,m), 8.38 (NH on 2, d, J=9.6 Hz), 1.98 (3, m), 0.92 (4, d, J=6.6 Hz), 1.40 (5, m), 1.13 (5, m), 0.88 (6, t, J=7.2 Hz); *Thr-1* 4.63 (2, t, J=9.3 Hz), 8.12 (NH on 2, d, J=5.7), 5.07 (3, dq, 9.6, 6.0 Hz), 1.18 (4, d, J=6.3 Hz); *Ileu-2* 4.52 (2, m), 7.72 (NH on 2, d, J=8.4 Hz), 1.88 (3, m), 0.88 (4, d, J=6.3 Hz), 1.40 (5, m), 1.13 (5, m), 0.88 (6, d, J=7.2 Hz); *Orn* 4.48 (2, m), 7.92 (NH on 2, d, J=7.8 Hz), 1.76 (3, m), 1.83 (4, m), 3.10 (5, p, J=5.1Hz); *Pro* 4.42 (2, m), 2.12 (3, m), 1.97 (3, m), 2.02 (4, m), 1.88 (4, m), 3.75 (5, m), 3.68 (5, m); *Val-3* 4.41 (2, m), 7.90 (NH on 2, d, J=7.2 Hz), 2.17 (3, m), 0.95 (4, m), 0.85 (5, m); *Val-4* 4.34 (2, m), 7.68 (NH on 2, d, J=8.1 Hz), 2.17 (3, m), 0.95 (4, m), 0.90 (5, m); *Thr-2* 4.46 (2, m), 7.77 (NH on 2, d, J=8.1), 4.21 (3, dq, 6.3, 3.6 Hz), 1.12 (4, d, J=6.6); *Val-5* 4.32 (2, m), 7.85, (NH on 2, d, J=8.1 Hz), 7.82 (NH on (second conformation), d, J=8.1 Hz), 2.14 (3, m), 0.95 (4, m), 0.90 (5, m); 5-*MeHex* 2.26 (2, m), 1.60 (3, m), 1.20 (4, m), 1.55 (5, m), 0.87 (6, d, J=7.2 Hz), 0.87 (7, d, J=7.2 Hz); 5-*MeHex* 2.29 (2,m), 1.65 (3, m), 1.40 (3, m), 1.13 (4, m), 1.35 (5, m), 0.90 (6, m), 0.90 (7, m); ^{13}C NMR (125 MHz TFA/DMF): amino acid unit, δ (carbon position); *Val-1* 170.40 (1), 60.31 (2), 30.75 (3), 19.58 (4), 18.76 (5); *Dhb* 164.54 (1), 130.30 (2), 131.26 (3), 12.68 (4); *Phe* 171.31 (1), 56.27 (2), 36.79 (3), 138.23 (4), 129.86 (5), 128.77 (6), 126.98 (7); *Val-2* 172.94 (1), 58.57 (2), 32.38 (3), 18.92 (4), 17.60 (5); *Ileu-1* 171.87 (1), 57.48 (2), 38.78 (3), 14.56 (4), 26.78 (5), 11.67; *Thr-1* 169.68 (1), 57.37 (2), 71.05 (3), 17.34 (4); *Ileu-2* 171.92 (1), 57.29 (2), 38.01 (3), 14.78 (4), 26.55 (5), 11.63 (6); *Orn* 172.01 (1), 52.87 (2), 29.63 (3), 24.39 (4), 40.05 (5); *Pro* 172.55 (1), 60.23 (2), 29.58 (3), 25.38 (4), 48.03 (5); *Val-3* 171.28 (1), 57.57 (2), 30.54(3), 19.61 (4), 18.80 (5); *Val-4* 171.83 (1), 59.10 (2), 31.26 (3), 19.45 (4), 18.08 (5); *Thr-2* 170.97 (1),, 58.89 (2), 67.36 (3), 19.66 (4); *Val-5* 172.67 (1), 59.64 (2), 30.66 (3), 19.61 (4), 18.43 (5); 5- *MeHex* 173.83 (1), 36.28 (2), 23.99 (3), 38.96 (4), 28.10 (5), 22.54 (6), 22.50 (7); *5-MeHex* (second conformation) 174.08 (1), 33.86 (2), 32.84 (3), 29.75 (4), 34.54 (5), 19.51 (6), 11.20 (7); IR neat (NaCl): 3287 (s, br), 2964 (s, br), 1646 (s), 1528 (s), 1465 (s), 1388 (m), 1228 (m), cm^{-1}; mass spectrum HRFAB *m/z* (fragment, %) 1477.9408 (M^+ + 1,85)(calcd for $C_{75}H_{125}N_{14}O_{16}$: 1477.9398); UV (MeOH): λ_{max} 204 (89,630)nm.

Amino acid analysis by GC-MS with a Chirasil-Val column indicates that Kahalalide F consists of D-Ileu, -Orn, L-Phe, D-Pro, L-Thr, D-Allo-Thr, 3 D-Val and 2 L-Val.

Table II ^{1}H and ^{13}C NMR Data for Kahalalide F (1) in DMF/TFA

Amino Acid	Carbon	^{13}C, ppm[a]	Mult.	^{1}H, ppm[b]	Multiplicity
Valine-1	1	170.4	s	(NH) 7.11	d, J=8.9
	2	60.3	d	4.16	t, J=9.0
	3	30.8	d	1.77	m
	4	19.6	q	0.95	m
	5	18.8	q	0.95	m
Dehydroamino -butyric acid	1	164.5	s	(NH) 9.20	s
	2	130.3	s		
	3	131.3	d	6.48	q, J=6.9
	4	12.7	q	1.43	d, J=6.6
Phenylalanine	1	171.3	s	(NH) 8.62	d, J=6.6
	2	56.3	d	4.68	q, J=6.6
	3	36.8	t	3.23	dd, J=13.7, 7.2
				3.00	dd, J=13.7, 9.0
	4	138.2	s		
	5, 5'	129.9	d	7.32	d, J=7.2
	6, 6'	128.8	d	7.28	t, J= 7.5
	7	127.0	d	7.21	t, J=7.2
Valine-2	1	172.9	s	(NH) 7.82	d, J=6.6
	2	58.6	d	4.36	m
	3	32.4	d	2.12	m
	4	18.9	q	0.85	m
	5	17.6	q	0.77	d, J=6.6
Isoleucine-1	1	171.9	s	(NH) 8.38	d, J=9.6
	2	57.5	d	4.53	m
	3	38.8	d	1.98	m
	4	14.6	q	0.92	d, J=6.6
	5	26.8	t	1.40, 1.13	m, m
	6	11.7	q	0.88	t, J=7.2
Threonine-1	1	169.7	s	(NH) 8.12	d, J=5.7
	2	57.4	d	4.63	t, J=9.3
	3	71.1	d	5.07	dq, J=9.6, 6.0
	4	17.3	q	1.18	d, J=6.3
Isoleucine-2	1	171.9	s	(NH) 7.72	d, J=8.4
	2	57.3	d	4.52	m
	3	38.0	d	1.88	m
	4	14.8	q	0.88	d, J=6.3
	5	26.6	t	1.40, 1.13	m, m
	6	11.6	q	0.88	t, J=7.2
Ornithine	1	172.0	s	(NH) 7.92	d, J=7.8
	2	52.9	d	4.48	m
	3	29.6	t	1.76	m
	4	24.4	t	1.83	m
	5	40.1	t	3.10	p, 5.1
Proline	1	172.6	s		
	2	60.2	d	4.42	m
	3	29.6	t	2.12, 1.97	m, m
	4	25.4	t	2.02, 1.88	m, m
	5	48.0	t	3.75, 3.68	m, m

Table II Continued

Valine-3	1	171.3	s	(NH) 7.90	d, J=7.2
	2	57.6	d	4.41	m
	3	30.5	d	2.12	m
	4	19.6	q	0.95	m
	5	18.8	q	0.85	m
Valine-4	1	171.8	s	(NH) 7.68	d, J=8.1
	2	59.1	d	4.34	m
	3	31.3	d	2.17	m
	4	19.5	q	0.95	m
	5	18.1	q	0.90	m
Threonine-2	1	171.0	s	(NH) 7.77	d, J=8.1
	2	58.9	d	4.46	m
	3	67.4	d	4.21	dq, J=6.3, 3.6
	4	19.7	q	1.12	d, J=6.6
Valine-5	1	172.7 conf. #2	s	(NH) 7.85, (NH) 7.82	d, J=8.1 d, J=8.1
	2	59.6	d	4.32	m
	3	30.7	d	2.14	m
	4	19.6	q	0.95	m
	5	18.4	q	0.90	m
5-Methyl - Hexanoic acid	1	173.8	s		
	2	36.3	t	2.26	m
	3	24.0	t	1.60	m
	4	39.0	t	1.20	m
	5	28.1	d	1.55	m
	6	22.5	q	0.87	d, J=7.2
	7	22.5	q	0.87	d, J=7.2
5-Methyl - Hexanoic acid (second conformation)	1	174.1	s		
	2	33.9	t	2.29	m
	3	32.8	t	1.65, 1.40	m
	4	29.8	t	1.13	m
	5	34.5	d	1.35	m
	6	19.5	q	0.90	m
	7	11.2	q	0.90	m

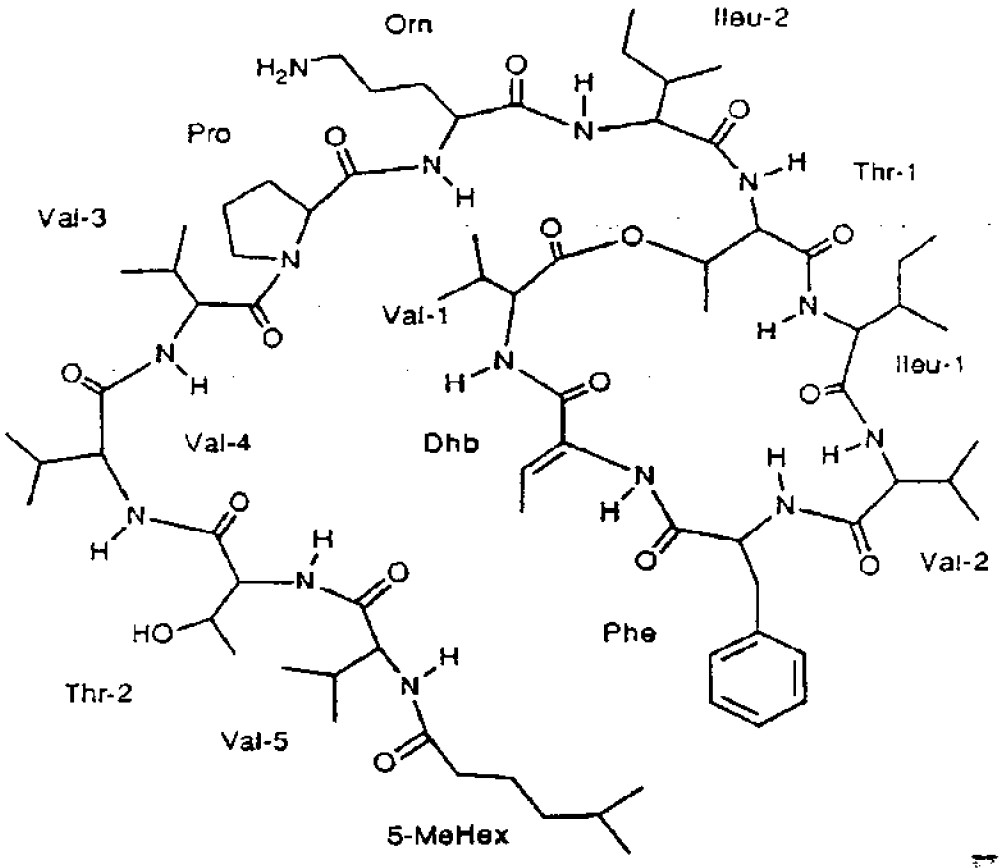
[a] at 125 MHz, DMF signal at 35.2 ppm; [b] at 500 MHz, DMF signal at 2.91 ppm.

Table I. *In vitro* Activity of Kahalalide F from *Elysia rufescens* Assay (M.I.C. μg/mL)

Cytotoxicity μg/mL (IC_{50})	
A-549	2.5
HT-29	0.25-0.5
Antiviral μg/mL (% reduction)	
Mv 1 Lu/HSV II	0.5 (95%)
CV-1/HSV-1	>8
BHK/VSV	>8
Antifungal 6mm disk	50 μg/disk
Aspergillus oryzae	19 mm
Penicillium notatum	26 mm
Tricophyton mentagrophy	34 mm
Saccharomyces cerevisiae	neg
Candida albicans	16 mm

Claims

1. Kalahide F of the formula: -



2. A pharmaceutical composition comprising Kalahide F in association with a pharmaceutical carrier or diluent.

3. The use of Kalahide F in the manufacture of an antitumor or antiviral pharmaceutical composition.

4. A method of treating hormones which comprises administering Kalahide F to a subject.

5. An antiviral method which comprises administering Kalahide F to a subject.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 30 0780

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	EP-A-0 358 418 (SANKYO COMPANY LIMITED) 14 March 1990 ---		C07K7/56 C07K7/06 A61K37/02
A	EP-A-0 399 685 (ARIZONA BOARD OF REGENTS) 28 November 1990 -----		

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C07K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	7 April 1994	Deffner, C-A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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